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Characterization of Extracellular Mn^{2+}-Oxidizing Activity and Isolation of an Mn^{2+}-Oxidizing Protein from *Leptothrix discophora* SS-1

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Supernatant fluid from *Leptothrix discophora* SS-1 cultures possessed high Mn^{2+}-ozidizing activity. Studies of temperature and pH optima, chemical inhibition, and protease sensitivity suggested that the activity may be enzymatic. Kinetic studies of unconcentrated supernatant fluid indicated an apparent K_m of 7 μM Mn^{2+} in the 1 to 200 μM Mn^{2+} range. The greatest V_{max} value observed was 1.4 nmol of Mn^{2+} oxidized min^{-1} μg of $protein^{-1}$ in unconcentrated samples. When the supernatant fluid was concentrated on DEAE-cellulose and the activity was eluted with $MgSO_4$, an Mn^{2+}-oxidizing protein was detected in the concentrate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Mn^{2+}-oxidizing protein appeared to have a molecular weight of 110,000 in 10% polyacrylamide gels and of 100,000 in 8% gels. Periodic acid-Schiff base staining of overloaded polyacrylamide gels showed that the DEAE-cellulose concentrate contained abundant high-molecular-weight polysaccharides; concurrent staining of the Mn^{2+}-oxidizing band suggested that it too contained carbohydrate components. Isolation of the protein was achieved by subjecting the DEAE-cellulose concentrate to Sephacryl gel filtration in the presence of 1% sodium dodecyl sulfate, followed by preparative electrophoresis and reverse-polarity elution. However, these procedures resulted in loss of a large proportion of the activity, which precluded recovery of the protein in significant quantity.

The biochemical mechanism of bacterial manganese oxidation has attracted the interest of microbiologists for many years. Much of this interest has centered on the possible use of Mn^{2+} as a source of energy during autotrophic or mixotrophic growth (for a recent review, see reference 7). The Mn^{2+}-oxidizing activity of *Leptothrix discophora* (also known as *Sphaerotilus discophorus*) and the influence of Mn^{2+} on its growth have been the object of numerous studies (2–4, 9, 11, 14, 15, 17). Ali and Stokes (4) showed that growth yields of *L. discophora* increased in the presence of Mn^{2+}; however, others (11, 15, 17, 19) questioned their claims of energy gain from Mn^{2+} oxidation. Mulder (15) and van Veen (17) instead proposed that *Leptothrix* spp. excreted proteinaceous factors which catalyzed Mn^{2+} oxidation in the sheath by a mechanism unrelated to the energy needs of the bacterium. Although these workers demonstrated heat and protease sensitivity of the extracellular activity, they neither isolated nor further characterized the proposed Mn^{2+}-oxidizing factors.

In our previous work (2, 3), we have shown that *L. discophora* SS-1 no longer forms a well-defined sheath, yet the bacterium retains the ability to catalyze a rapid extracellular oxidation of Mn^{2+} to MnO_x, where x is at least 1.65 (L. F. Adams, Ph.D. dissertation, Cornell University, Ithaca, N.Y., 1986). Furthermore, we have shown (2) that Mn^{2+} oxidation does not support mixotrophic or autotrophic growth; instead, all concentrations of Mn^{2+} added to a heterotrophic medium inhibited the growth of this strain. In the present work, we report characteristics of the extracellular Mn^{2+}-oxidizing activity of SS-1, as revealed by pH, temperature, inhibition, and kinetic studies on cell-free spent culture medium. In addition, we describe procedures that resulted in the isolation of a Mn^{2+}-oxidizing protein. Our results are consistent with those recently obtained by another laboratory (5) for the same strain of *L. discophora*.

(Preliminary reports of this work have been presented [8; L. F. Adams and W. C. Ghiorse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, N8, p. 218].)

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff base; MW, molecular weight.

Bacterial culture, media, and growth conditions. *L. discophora* SS-1 (ATCC 43182) was maintained on peptone-yeast extract-glucose broth (2). For the experiments described below, cells were grown in 100 ml of unbuffered peptone-yeast extract-glucose medium in 250-ml Erlenmeyer flasks. Flasks were incubated at 25°C on a GIO Gyrotory shaker (New Brunswick Scientific Co.) at 150 rpm. To obtain a sufficient amount of culture fluid for isolation of Mn^{2+}-oxidizing protein, cultures were scaled up to a total volume of approximately 50 liters in three 20-liter Pyrex carboys, each filled with 16 liters of unbuffered peptone-yeast extract-glucose medium. The carboys were autoclaved, cooled overnight, and inoculated with 2 liters of a late-exponential-

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phase culture of SS-1. Carboys were then fitted with sterile air lines and bubbled vigorously with filter-sterilized air for 24 to 36 h at room temperature (22 to 25°C).

Assays for Mn^{2+}-oxidizing activity. Filtration assays were performed by diluting 0.5 ml of sample to 10 ml with 15 mM HEPES buffer (pH 7.3) to give a final protein concentration of 25 μg ml^{-1}. Mn^{2+} oxidation was initiated by adding 100 μM $MnSO_4$ to the assay mixture and incubating the mixture. At zero time, and at intervals thereafter for periods of up to 3 h, 2-ml (at later time points, 0.5-ml) samples were removed and filtered through a 25-mm Metricel GA-8 (pore size, 0.2-μm) membrane filter (Gelman Sciences, Inc., Ann Arbor, Mich.). Filtration was followed by a 200-μl deionized-water rinse to remove residual Mn^{2+} (2). The amount of Mn in the brown oxide (MnO_x) product trapped on the filter was then measured by atomic absorption spectrophotometry as described previously (2).

Spectrophotometric assays were performed by placing HEPES-buffered samples in 0.4-ml quartz microcuvettes and (except for kinetic studies) adding 200 μM $MnSO_4$ in a final volume of 0.3 ml. MnO_x production was monitored in reaction cuvettes by measuring the increase in A_{366} (12) in a recording spectrophotometer (model 260; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Reference cuvettes contained no $MnSO_4$. Occasionally, after several assays were repeated in the same cuvettes, a visible film of brown oxide formed on the walls of the reaction cuvettes. The film was removed by a 5-min soak in 1 N HCl followed by careful rinsing in deionized water. Measurements of absorbance by spectrophotometry and of Mn concentration by atomic absorption spectrophotometry (2) established a molar absorption coefficient at 366 nm of 9.1×10^3 (1-cm light path) for the oxide produced by strain SS-1. This value agrees closely with the value of 10^4 reported by Jung and Schweisfurth (12) for the manganic oxide produced by *Pseudomonas* sp. strain MnB1. The relationship between A_{366} and MnO_x concentration remained strictly linear to approximately 200 μM MnO_x. Oxidation of greater molar concentrations of Mn^{2+} resulted in precipitation of the oxide and loss of linearity.

Except for temperature optimum experiments, all assays were performed at room temperature.

Concentration of Mn^{2+}-oxidizing activity on DEAE-cellulose. Mn^{2+}-oxidizing activity was concentrated from 40 to 50 liters of late-exponential-phase *L. discophora* cultures by centrifugation at 15,000 × *g* and filtration of the supernatant fluid through a 27-cm-diameter Büchner funnel containing approximately 600 ml of wet DEAE-cellulose (Sigma Chemical Co., St. Louis, Mo.) supported by a 25-cm-diameter Whatman no. 1 filter paper disk. The loaded cellulosic slurry was then poured into a 5-cm-diameter column (30-cm bed height and 590-ml initial bed volume) and eluted at 5 ml min^{-1} with 1,200 ml of a 75 to 250 mM linear gradient of $MgSO_4$ at 4°C. Active fractions, detected by the spectrophotometric assay, were dialyzed at 4°C against two 100-fold volumes of the 15 mM HEPES buffer and then concentrated either by placement of the dialysis tubes on dry Aquacide II (carboxymethyl cellulose, 500,000 MW; Calbiochem-Behring, La Jolla, Calif.) or by vacuum dialysis (MPDC-320 unit, 60,000 MW cutoff; BioMolecular Dynamics, Beaverton, Oreg.).

Determination of pH and temperature optima. The optimal pH for Mn^{2+} oxidation was determined by adjusting 300 mM stock solutions of MES, PIPES, HEPES, and EPPS buffers to pH values within 1.5 U of their respective pK_a values and by adding the solutions to culture supernatant or diluted DEAE-cellulose concentrate at a final buffer concentration of 30 mM. Mn^{2+}-oxidizing activity was determined by the spectrophotometric assay.

The optimal temperature for Mn^{2+} oxidation was determined by dilution of culture supernatant or concentrate with the 15 mM HEPES buffer and transfer of 0.3-ml portions to 0.4-ml quartz thermal cuvettes. The cuvettes were incubated in the water-jacketed cuvette holder of the Gilford 260 spectrophotometer at the desired temperature ± 0.1°C for 10 min. $MnSO_4$ was then added, and Mn^{2+}-oxidizing activity was determined by the spectrophotometric assay.

Effects of chemical inhibitors and enzymes on Mn^{2+}-oxidizing activity. KCN, *o*-phenanthroline, $HgCl_2$, and NaN_3 were dissolved in deionized water; inhibition was determined by comparing the activity of amended and unamended samples with the filtration assay. EDTA and sulfate salts of Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} were dissolved in the 15 mM HEPES buffer; inhibition was determined by the spectrophotometric assay.

The effects of trypsin, lysozyme, and RNase (Millipore Corp., Freehold, N.J.) and of hyaluronidase, pronase E (*Streptomyces griseus*), and proteinase K (*Tritirachium album*) (Sigma) were determined by adding solutions of the enzymes to 10 ml of the 15 mM HEPES-buffered reaction mixture to a final concentration of 25 μg of enzyme ml^{-1} and then adjusting the mixture to the pH indicated by the supplier as optimal for the activity of the enzyme. The enzyme mixtures were incubated at room temperature for 30 min, readjusted to pH 7.3, and assayed by the filtration method. Boiled enzyme preparations were employed in control tubes.

Kinetic studies. For kinetic studies, concentrated stock solutions of $MnSO_4$ were mixed with HEPES-buffered samples in microcuvettes to final concentrations ranging from 1 μM to 2 mM Mn^{2+}. Linear oxidation rates were recorded only for the first 10 min, which generally corresponded to oxidation of less than 20% of the total available Mn^{2+}.

SDS-PAGE. Slab gels (0.75 by 140 by 140 mm) were formed with 6, 8, or 10% acrylamide and overlaid with a 3% acrylamide stacking gel of 2-cm height, by the general procedure of Laemmli (13). Wells of individual lanes were loaded with 30 μl of sample mixed with an equal volume of treatment buffer containing 4% SDS, 20% glycerol, and a trace of bromophenol blue as the marker dye. β-Mercaptoethanol was not added to the treatment buffer, nor were samples boiled, as both of these treatments abolished Mn^{2+}-oxidizing activity. High-MW marker proteins (Bio-Rad Laboratories, Richmond, Calif.) were diluted 300-fold with a mixture of equal volumes of treatment and tank buffer before being loaded. Electrophoresis was performed at 4°C at 1.5 mA of constant current per cm for approximately 2.5 h. After electrophoresis, gels were stained in a solution of 0.05% Coomassie brilliant blue G-250 in 25% (vol/vol) isopropanol and 10% (vol/vol) acetic acid or with a commercial silver stain kit (Bio-Rad). Both protein-staining procedures were used successfully without modification on gels that had been stained previously for Mn^{2+}-oxidizing activity (see below). Some gels were also stained for glycoprotein and polysaccharide by the PAS method of Zacharius et al. (19). Samples destined for PAS staining were concentrated by precipitation with a twofold greater volume of 95% ethanol or 100% acetone in a methanol-dry ice bath for 30 min. After evaporation to dryness, the precipitate was suspended in a smaller volume of the 15 mM HEPES buffer and loaded in wells for electrophoresis in the usual manner.

Staining for Mn^{2+}-oxidizing activity in SDS-polyacrylamide gels. Gels were prepared for activity staining after SDS-

PAGE by incubation in 200 ml of 2.5% (vol/vol) Triton X-100 for 30 min with gentle agitation, followed by thorough washing for an additional 30 min in deionized water to completely remove SDS–Triton X-100 mixed micelles (10). Gels were then soaked in 200 ml of 100 or 200 μM $MnSO_4$ in the 15 mM HEPES buffer until the characteristic brown, MnO_x bands appeared (usually within 15 min). All staining steps were done at room temperature.

Isolation of Mn^{2+}-oxidizing protein. From active, DEAE-cellulose-concentrated fractions, 2- to 7-ml samples were applied to a column (2.5 by 30 cm) of Sephacryl SF-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) and eluted with 1% SDS in the 15 mM HEPES buffer at 0.5 ml min^{-1}. Active fractions were identified by SDS-PAGE activity staining and then concentrated with simultaneous removal of SDS by either vacuum dialysis or passage through Centricon 10 or 30 microconcentrators (Amicon Corp., Danvers, Mass.). If the protein concentration of samples was less than 1 μg ml^{-1} the Centricon units were pretreated with 20 μl of a solution of chicken egg albumin (400 μg ml^{-1}; Sigma) to hinder adsorption of activity. Mn^{2+}-oxidizing protein was isolated from a pool of concentrated active fractions by loading the concentrate into a one-well, 1.5-mm-thick preparative polyacrylamide slab gel. After electrophoresis, active bands were subjected to reverse-polarity elution to recover the proteins (1). Active bands were located before reverse-polarity elution by washing the preparative gel with 2.5% Triton X-100 as described above and incubating in 100 μM $MnSO_4$ until the characteristic brown MnO_x bands were just barely visible.

Protein determinations. Duplicate samples were diluted to a total volume of 0.8 ml, and 0.2 ml of the undiluted Bio-Rad reagent was added to each sample (microassay procedure) (6). The A_{595} was measured. Bovine serum albumin was the standard.

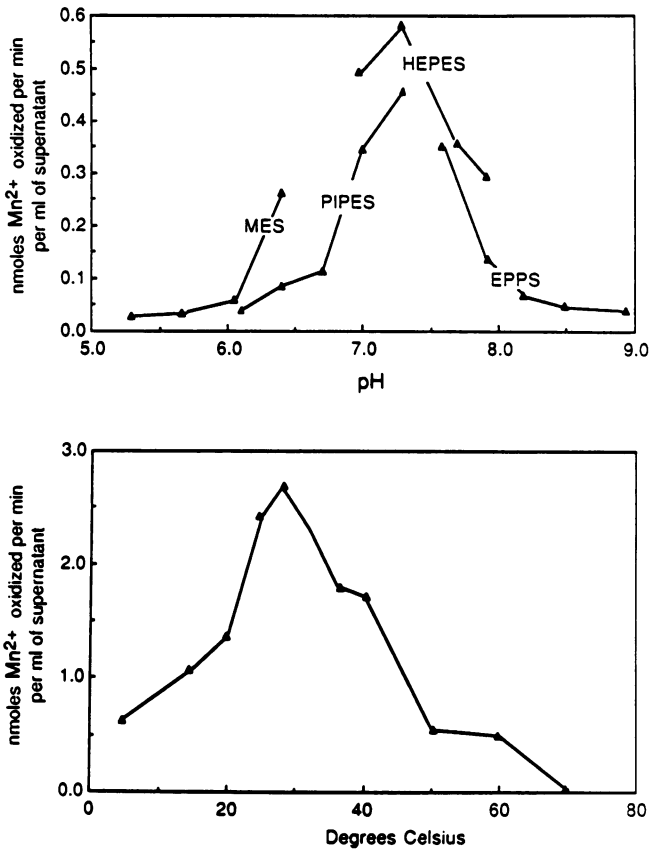

FIG. 1. Profile of Mn^{2+}-oxidizing activity in culture supernatant as a function of pH and temperature.

RESULTS

Temperature and pH optima. The Mn^{2+}-oxidizing activity of unconcentrated supernatant exhibited well-defined temperature and pH optima at approximately 28°C and pH 7.3, respectively (Fig. 1). DEAE-cellulose-concentrated supernatant exhibited nearly identical profiles (data not shown).

Kinetics of Mn^{2+} oxidation. Kinetic parameters for the Mn^{2+}-oxidizing activity were determined on both unconcentrated and DEAE-cellulose-concentrated supernatant fluid (Table 1) by measuring initial oxidation rates over a wide range of Mn^{2+} concentrations (Fig. 2). Although a large portion of activity was lost during initial concentration on DEAE-cellulose (Table 1), Mn^{2+}-oxidizing activity in both types of preparations approximated the Michaelis-Menten kinetic model up to 200 μM Mn^{2+}. Mn^{2+}-oxidizing activity was inhibited by Mn^{2+} concentrations above 400 μM; it was completely eliminated by 2 mM Mn^{2+} (Fig. 2). In the 1 to 200 μM range, apparent K_m values for unconcentrated samples averaged 7.0 ± 3.2 μM (±1 standard deviation, $n = 5$). For concentrated samples, the mean value was approximately the same, but the standard deviation was greater. V_{max} values up to 1.4 nmol of Mn^{2+} oxidized min^{-1} μg of $protein^{-1}$ were observed for unconcentrated samples. DEAE-cellulose-concentrated samples lost much of their specific activity (Table 1); thus, their V_{max} values were correspondingly lower.

Inhibition of Mn^{2+}-oxidizing activity. Both unconcentrated and concentrated sources of activity were sensitive to cyanide, *o*-phenanthroline, and mercuric chloride (Table 2). Although CN^- may complex Mn^{2+} and thus interfere with its oxidation (16), the MICs of CN^- (1 to 10 μM) were far less than the amount of Mn^{2+} (200 μM) used to initiate oxidation in these assays, indicating that Mn^{2+} complexation did not play a major role in CN^- inhibition. The same argument can be applied to inhibition by *o*-phenanthroline. Azide did not substantially inhibit Mn^{2+}-oxidizing activity at concentrations below 1 mM.

Mn^{2+}-oxidizing activity also was inhibited by 10 μM Zn^{2+} and Co^{2+} and by 100 μM Cu^{2+} and Ni^+ (data not shown). EDTA inhibited Mn^{2+}-oxidizing activity only at concentrations in excess of the Mn^{2+} concentration in the assay mixture, indicating that its inhibitory effect probably resulted from chelation of Mn^{2+}. Mn^{2+}-oxidizing activity was insensitive to digestion with trypsin, proteinase K, lysozyme, RNase, and hyaluronidase. However, incubation with 25 μg pronase E ml^{-1} for 30 min eliminated nearly all activity in unconcentrated supernatant (data not shown).

Detection of Mn^{2+}-oxidizing protein by SDS-PAGE. Attempts to separate Mn^{2+}-oxidizing factors from concentrated samples by PAGE without detergents failed. In detergent-free PAGE, Mn^{2+}-oxidizing activity of DEAE-cellulose-concentrated supernatant fluids was visualized only as long, brown smears in the upper region of the polyacrylamide gel. Furthermore, Mn^{2+}-oxidizing activity could not be detected in polyacrylamide gels containing 0.1% SDS, which completely inhibited its activity. However, treatment of the SDS-containing gels to remove SDS followed by soaking the gel in 200 μM $MnSO_4$ solution

TABLE 1. Procedure for concentrating Mn^{2+}-oxidizing activity and isolating an Mn^{2+}-oxidizing protein from supernatant fluid

Step	Volume (ml)	Total activity (nmol of Mn^{2+} oxidized min^{-1})	% Recovery	Total protein (μg)	Sp act (nmol of Mn^{2+} oxidized min^{-1} μg of $protein^{-1}$)
23,000 × *g* supernatant (unconcentrated)	52,000	92,860	100	78,000	1.19
DEAE-cellulose anion exchange (large peak of activity in eluate)	21	5,720	6.2	28,900	0.20
Sephacryl SF-200 gel filtration in 1% SDS (pool of several active fractions)[a]	1.2	1,090	1.2	3,600	0.30
Preparative electrophoresis and reverse-polarity elution	2.0	0.40	0.0004	32	0.01

[a] Activity was detected in individual fractions in the presence of 1% SDS; however, SDS was removed by pooling active fractions and dialyzing against 15 mM HEPES (pH 7.3) to obtain the activity measurements listed.

permitted detection of the activity, usually within minutes. Preliminary work (8) established that parallel lanes of the same polyacrylamide gel could be stained with Coomassie blue or silver to show faint protein bands at the same apparent MW as the Mn^{2+}-oxidizing bands.

The Mn^{2+}-oxidizing protein had an apparent MW of 110,000 in 10% polyacrylamide gels (Fig. 3 and 4) and 100,000 in 6 and 8% gels. We did not obtain sufficient quantities of pure protein to obtain an MW by alternative methods. A very faint gray-brown band appeared occasionally at approximately 57,000 MW in 10% gels (or 52,000 in lower-percentage gels). The lower-MW band may represent a subunit or degradation product of the larger-MW protein. In preparations stored for more than 3 days at 4°C, the major Mn^{2+}-oxidizing band appeared in the form of a 110,000- and 106,000-MW doublet in 10% polyacrylamide gels (Fig. 3) or as a 100,000- and 85,000-MW pair in lower-percentage gels (data not shown). The lower band of the pair was generally less active in fresh samples. However, after storage for 30 days or longer at 4°C, nearly all activity appeared at the lower position, as did the activity from fractions that had been repeatedly frozen and thawed.

Isolation of Mn^{2+}-oxidizing protein. Batch anion exchange onto DEAE-cellulose permitted a substantial concentration of Mn^{2+}-oxidizing activity from the culture supernatant, but resulted in a sixfold loss in specific activity (Table 1). Concentration by ultrafiltration and dialysis techniques was also tried, but it resulted in an even greater loss of specific activity. After DEAE-cellulose concentration, a large peak containing most of the remaining activity eluted from the column at 200 mM $MgSO_4$ (Table 1). This peak contained a very large number of proteins, as indicated by SDS-PAGE (Fig. 4). Determination of total carbohydrate content by the phenol-sulfuric acid method also indicated a two- to fivefold-greater quantity of carbohydrate over protein in this peak. The loss of specific activity and the likelihood of aggregation indicated by elution of a single peak of activity suggested that the DEAE-cellulose column failed to separate protein and polysaccharide components in the supernatant fluid. Subsequent gel filtration with a Bio-Rad P-300 column did not further resolve these components. Nearly all Mn^{2+}-oxidizing activity eluted from the column immediately after the void volume, accompanied by quantitative recovery of the protein added to the column. Negative staining and transmission electron microscopy of the active fractions from the Bio-Rad P-300 column revealed extensive, membranous aggregates consisting of particles ranging from 25 to 50 nm in diameter (data not shown). These particles and aggregates were morphologically similar to the outer membrane blebs seen in negative stains of whole *L. discophora* cells (3).

A number of detergents and disaggregating reagents were tested in an attempt to separate the Mn^{2+}-oxidizing protein from the protein-carbohydrate-outer membrane aggregates in the DEAE-cellulose concentrate. Sucrose density gradient centrifugation or gel filtration in the presence of 6 M guanidine HCl and urea caused irreversible loss of Mn^{2+}-

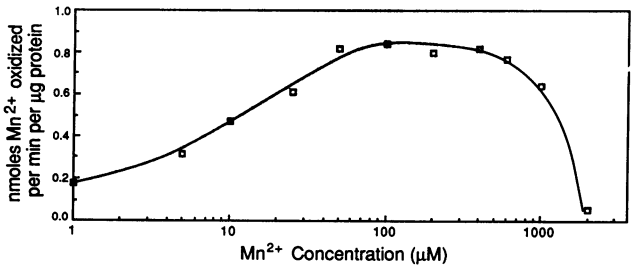

FIG. 2. Initial rates of Mn^{2+} oxidation as a function of Mn^{2+} concentration added to unconcentrated supernatant fluid.

TABLE 2. Effect of chemical inhibitors on Mn^{2+}-oxidizing activity[a]

Compound and concn (μM)	nmol of Mn^{2+} oxidized min^{-1} ml^{-1}
No addition	0.82
KCN	
1	0.71
100	<0.02
o-Phenanthroline	
1	0.71
10	0.47
100	<0.02
$HgCl_2$	
1	0.88
10	0.53
100	<0.02
NaN_3	
1,000	0.19

[a] DEAE-cellulose-concentrated supernatant fluid was added to test tubes at 25 μg ml^{-1}. Mn^{2+} oxidation rates were determined by the filtration assay described in Materials and Methods.

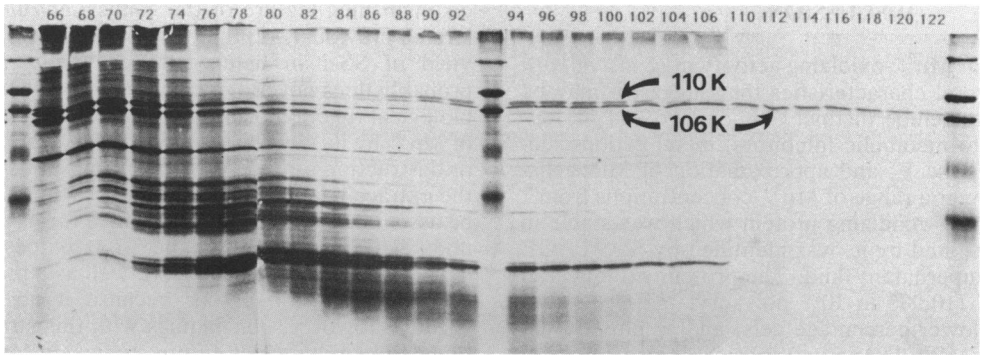

FIG. 3. Composite electrophoretogram (SDS-10% PAGE) showing sequential, even-numbered fractions from a Sephacryl SF-200 gel filtration column eluted with 1% SDS in 15 mM HEPES, pH 7.3. Note retention of the 110,000- (110K) and 106,000 (106K)-MW Mn^{2+}-oxidizing bands until most of the other proteins were eluted from the column. The gels were first stained with $MnSO_4$ for Mn^{2+}-oxidizing activity and then with silver to reveal protein bands. Unmarked lanes at the ends and at the center were loaded with MW standards as follows: myosin (200,000 MW), β-galactosidase (116,250), phosphorylase *b* (92,500), bovine serum albumin (66,200), and ovalbumin (45,000).

oxidizing activity. Detergent solutions such as 2% Triton X-100, 1% deoxycholate, and 1% cholate improved fractionation, but also eliminated activity.

Separation of an Mn^{2+}-oxidizing protein from other proteins finally was achieved by adding 1% SDS to DEAE-cellulose concentrate and applying the mixture to a Sephacryl SF-200 gel filtration column. The Sephacryl column was then eluted with 1% SDS in 15 mM HEPES buffer. The Mn^{2+}-oxidizing protein did not elute according to its MW (Fig. 3). Instead, it was retained on the Sephacryl column until most other proteins—of both high and low MW—had eluted. The reason for this retention is not known, but the pattern shown in Fig. 3 was highly reproducible.

Pooling and concentration of the most active fractions from the SDS-Sephacryl gel filtration step resulted in a preparation containing approximately 1.5 times more specific activity than the DEAE-cellulose concentrate did (Table 1). At this stage, the SDS-stable Mn^{2+}-oxidizing protein was separated from most other proteins (Fig. 4). It was identified as two close-running bands at 110,000 and 106,000 MW (Fig. 3 and 4). Mixing of inactive and active fractions from the Sephacryl gel filtration column did not increase activity.

Preparative electrophoresis of pooled SDS-Sephacryl gel filtration fractions followed by reverse-polarity elution of the active bands resulted in the isolation of a very small amount of active material primarily containing the 106,000-MW band (Fig. 4). These final procedures appeared to convert the 110,000-MW protein to the 106,000-MW form. In addition, these final steps resulted in a drastic (30-fold) loss of specific activity (Table 1). Nevertheless, a single Mn^{2+}-oxidizing protein band essentially free of other proteins was obtained (Fig. 4).

PAS staining of the Mn^{2+}-oxidizing protein. To further characterize the Mn^{2+}-oxidizing activity, the DEAE cellulose concentration was subjected to cold-ethanol or -acetone precipitation. PAS staining after SDS-PAGE revealed the presence of abundant high-MW polysaccharides. When large amounts of the precipitate were deliberately overloaded in SDS-PAGE gels, a PAS-positive band appeared at 100,000 MW in 8% gels (Fig. 5) and 110,000 MW in 10% gels. In some instances, a faint PAS-positive band also appeared at the 52,000-MW position in 8% gels (57,000 MW in 10% gels) (data not shown).

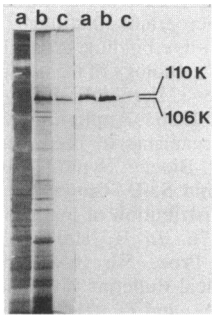

FIG. 4. Electrophoretogram (SDS-10% PAGE) showing the proteins present after each isolation step described in Table 1. The left and right sides of the gel were loaded with the same volume of sample; the left side was stained with silver for protein, and the right side was treated as described in Materials and Methods to remove SDS and then stained with $MnSO_4$ to reveal Mn^{2+}-oxidizing bands. Lanes: a, high-activity peak from DEAE-cellulose column; b, active fraction pool obtained by Sephacryl SF-200 gel filtration; c, after preparative electrophoresis and reverse-polarity elution. Most Mn^{2+}-oxidizing protein and activity appear at the lower 106,000 (106K)-MW position after the preparative electrophoresis step represented in lanes c.

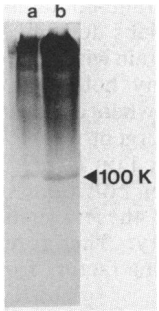

FIG. 5. Electrophoretogram (SDS-8% PAGE) showing PAS-stained contents of DEAE-cellulose-concentrated supernatant fluid. Lanes were deliberately overloaded with acetone-precipitated concentrate to reveal the PAS-staining bands at 100,000 (100K) MW. Lanes: a, 11 μg of protein; b, 26 μg of protein.

DISCUSSION

The extracellular Mn^{2+}-oxidizing activity in *L. discophora* SS-1 exhibits several characteristics that suggest it may be enzymatic. These include distinct temperature and pH optima; sensitivity to metabolic inhibitors, metal cations, detergents, and pronase E; and approximation of Michaelis-Menten kinetics over a range of Mn^{2+} concentrations from 1 to 200 μM. An Mn^{2+}-oxidizing protein which was stable in SDS, but also inhibited by it, was identified by SDS-PAGE in concentrated supernatant fluid. The protein exhibited an apparent MW of 110,000 in 10% polyacrylamide gels (but 100,000 MW on lower-percentage gels) and apparently degraded to a lower-MW, (but active) species. It also appeared to be associated with polysaccharide components in the supernatant fluid. These results are in agreement with those of other workers who have independently studied Mn^{2+}-oxidizing activity of strain SS-1 (5).

Based on our present results, we can conclude that at least part of the Mn^{2+}-oxidizing activity exhibited by this strain depends on the Mn^{2+}-oxidizing protein. However, it is possible that other, as yet unidentified, Mn^{2+}-oxidizing factors are produced by SS-1. Indeed, our finding that a large portion of the activity was lost in the first concentration step (Table 1) suggests that unidentified factors in the supernatant fluid were lost or inactivated at this step.

In addition to the problems alluded to in this paper, we encountered several other difficulties during attempts to purify the Mn^{2+}-oxidizing protein from SS-1 cell suspensions and concentrated supernatant fluid (Adams, Ph.D. dissertation). Our experience along these lines suggests that the Mn^{2+}-oxidizing protein probably is bound in aggregates, perhaps associated with outer membrane blebs (3) and extracellular polysaccharides. In keeping with this idea are results reported by earlier workers. For example, Mulder (15) noted mucilaginous material associated with the putative Mn^{2+}-oxidizing protein of *Leptothrix* spp. Also, Mills and Randles (14) reported high levels of Mn^{2+}-oxidizing activity in the 48,000 × *g* pellet of disrupted *L. discophora* cells, implying association of the activity with membrane aggregates. When we disrupted SS-1 cells, we found no Mn^{2+}-oxidizing activity in cytoplasmic fractions. Separation of membranes on a sucrose density gradient showed only minor amounts of Mn^{2+}-oxidizing activity in a plasma membrane band. Most of the Mn^{2+}-oxidizing activity, widely distributed through the gradient, apparently associated with outer membrane-polysaccharide aggregates of various densities (Adams, Ph.D. dissertation). Electron microscopy of active material from the sucrose density gradients confirmed the presence of membrane-containing aggregates similar to the outer membrane blebs described earlier. Association of the Mn^{2+}-oxidizing protein with outer membrane-exopolysaccharide aggregates may help to explain the smearing of activity we observed when concentrated supernatant fluid was subjected to detergent-free PAGE. Membrane blebs also have been observed on *L. discophora* SS-1 cells by de Vrind-de Jong and her co-workers (personal communication). We suspect that these structures may be carriers of Mn^{2+}-oxidizing activity. Therefore, future strategies for purifying Mn^{2+}-oxidizing factors from SS-1 must take them into account.

Our characterization of the extracellular Mn^{2+}-oxidizing activity of *L. discophora* SS-1 helps explain how this sheathless strain can oxidize Mn^{2+}; however, such an explanation does not permit us to ascribe a specific biological function to Mn^{2+} oxidation, which along with sheath formation is a distinguishing feature of the genus *Leptothrix*. We demonstrated previously (2) that Mn^{2+} inhibits the growth rate and yield of SS-1 in batch cultures. Thus, Mn^{2+} oxidation probably does not provide energy for growth. A more likely biological function of Mn^{2+} oxidation may be detoxification of Mn^{2+} by its conversion to insoluble MnO_x outside the cell or destruction of toxic oxygen species in its environment by the extracellular oxide (7). In nature, these functions would be associated with the sheath, which forms a tubelike matrix upon which manganese (and iron) oxides are deposited. Obviously, SS-1 does not serve well as a model of naturally occurring *Leptothrix* spp. because it no longer forms a sheath. However, our findings with this strain do provide a foundation upon which future studies on Mn^{2+} oxidation by sheathed strains of *Leptothrix* and other ferromanganese-depositing bacteria can be based.

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LITERATURE CITED

1. **Abramovitz, A. S., V. Randolph, A. Mehra, and S. Christakos.** 1984. Recovery of native proteins from preparative electrophoresis gel slices by reverse polarity elution. Prep. Biochem. **14:** 205–221.
2. **Adams, L. F., and W. C. Ghiorse.** 1985. Influence of manganese on growth of a sheathless strain of *Leptothrix discophora*. Appl. Environ. Microbiol. **49:**556–562.
3. **Adams, L. F., and W. C. Ghiorse.** 1986. Physiology and ultrastructure of *Leptothrix discophora* SS-1. Arch. Microbiol. **145:** 126–135.
4. **Ali, S. H., and J. L. Stokes.** 1971. Stimulation of heterotrophic and autotrophic growth of *Sphaerotilus discophorus* by manganous ions. Antonie van Leeuwenhoek J. Microbiol. Serol. **37:** 519–528.
5. **Boogerd, F. C., and J. P. M. de Vrind.** 1986. Manganese oxidation by *Leptothrix discophora*. J. Bacteriol. **169:**489–494.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Ghiorse, W. C.** 1984. Biology of iron- and manganese-depositing bacteria. Annu. Rev. Microbiol. **38:**515–550.
8. **Ghiorse, W. C.** 1986. Applicability of ferromanganese-depositing microorganisms to industrial metal recovery processes. Biotechnol. Bioeng. Symp. **16:**141–148.
9. **Ghiorse, W. C., and S. D. Chapnick.** 1983. Metal-depositing bacteria and the distribution of manganese and iron in swamp waters, p. 367–376. *In* R. Halberg (ed.), Environmental biogeochemistry. Proc. 5th Int. Symp. Environ. Biogeochemistry, Ecological Bulletins (Stockholm), vol. 35.
10. **Granelli-Piperno, A., and E. Reich.** 1978. A study of proteases and protease inhibitor complexes in biological fluids. J. Exp. Med. **148:**223–234.
11. **Hajj, H., and J. Makemson.** 1976. Determination of growth of *Sphaerotilus discophorus* in the presence of manganese. Appl. Environ. Microbiol. **32:**699–702.
12. **Jung, W. K., and R. Schweisfurth.** 1979. Manganese oxidation by an intracellular protein of a *Pseudomonas* species. Z. Allg. Mikrobiol. **19:**107–115.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Mills, V. H., and C. I. Randles.** 1979. Manganese oxidation in

Sphaerotilus discophorus particles. J. Gen. Appl. Microbiol. **25:** 205–207.

15. **Mulder, E. G.** 1972. Le cycle biologique tellurique et aquatique du fer et du manganèse. Rev. Écol. Biol. Sol **9:**321–348.
16. **Rosson, R. A., B. M. Tebo, and K. H. Nealson.** 1984. Use of poisons in determination of microbial manganese binding rates in seawater. Appl. Environ. Microbiol. **47:**740–745.
17. **van Veen, W. L.** 1972. Factors affecting the oxidation of manganese by *Sphaerotilus discophorus*. Antonie van Leeuwenhoek J. Microbiol. Serol. **38:**623–626.
18. **Van Veen, W. L., E. G. Mulder, and M. H. Deinema.** 1978. The *Sphaerotilus-Leptothrix* group of bacteria. Microbiol. Rev. **42:** 329–356.
19. **Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock.** 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **31:**148–152.